

# Host cyclophilin A mediates HIV-1 attachment to target cells via heparans

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**The present study proposes a novel mode of action for cyclophilin A (CypA) in the HIV-1 life cycle. We demonstrate that CypA-deficient viruses do not replicate because they fail to attach to target cells. We show that CypA is exposed at the viral membrane and mediates HIV-1 attachment. We identify heparan as the exclusive cellular binding partner for CypA. Furthermore, CypA binds directly to heparan via a domain rich in basic residues similar to known heparin-binding motifs. This interaction between exposed CypA and cell surface heparans represents the initial step of HIV-1 attachment and is a necessary precursor to gp120-binding to CD4. In conclusion, HIV-1 attachment to target cells is a multi-step process that requires an initial CypA–heparan interaction followed by the gp120–CD4 interaction.**

**Keywords:** attachment/cyclophilin A/heparan/human immunodeficiency virus type-1

## Introduction

The human immunodeficiency virus type-1 (HIV-1) requires the viral incorporation of host protein cyclophilin A (CypA) for replication (Franke *et al.*, 1994; Thali *et al.*, 1994). CypA was originally discovered as a specific ligand for the immunosuppressive drug, cyclosporin A (CsA) (Handschumacher *et al.*, 1984). CypA is an abundant cytosolic protein ubiquitously expressed in eukaryotic cells (Ryffel *et al.*, 1991). Despite its broad phylogenetic distribution, the physiological function of CypA has not yet been elucidated. Because of its peptidyl–prolyl *cis–trans* isomerase activity, CypA has been proposed to act as a chaperone in protein trafficking or as a catalyst of protein folding (Gething and Sambrook, 1992).

CypA is packaged into nascent HIV-1 particles by specifically binding to the capsid (CA) region of the Gag precursor at the time of viral assembly (Franke *et al.*, 1994; Thali *et al.*, 1994; Colgan *et al.*, 1996). Crystal structure analysis reveals that a proline-rich stretch of the single exposed loop of HIV-1 CA interacts with the catalytic domain of the isomerase (Gamble *et al.*, 1996). This interaction can be competitively disrupted by CsA (Luban *et al.*, 1993; Thali *et al.*, 1994; Franke and Luban, 1996). Preventing CypA packaging, either by the introduction of mutations in the binding region of CA, or

by the addition of CsA to particle producer cells, abrogates virus infectivity, demonstrating a requirement for CypA in HIV-1 replication (Franke *et al.*, 1994). Importantly, these CypA-deficient viruses are identical to wild-type particles by biochemical, enzymatic and electron microscopic analyses (Braaten *et al.*, 1996). To date, the block of infectivity of CypA-deficient virus has been attributed to events prior to the initiation of the viral reverse transcription into target cells (Braaten *et al.*, 1996).

HIV-1 CA forms the coat that protects and surrounds the viral genome. Fractionation studies of infected cells suggest that shortly after entry, CA separates from the viral nucleoprotein complex (Farnet and Haseltine, 1991). The observations that dissociation of CA from the viral nucleoprotein complex is required for productive infection, and that CypA is necessary for the initiation of reverse transcription, have prompted researchers to propose that CypA promotes HIV-1 uncoating in a manner similar to that of the chaperone heat shock protein (hsp) in the uncoating of clathrin from coated vesicles (Luban, 1996). In this model, CypA interferes with CA–CA interactions by binding to a single exposed HIV-1 CA loop, facilitating the breakdown of the shell of CA molecules that surrounds the viral genome. However, to date, no experimental evidence has been reported to support this model. Furthermore, recent studies demonstrate that CypA does not influence HIV-1 assembly, maturation or core stability at physiological concentrations, suggesting that CypA does not serve as an uncoating factor (Grättinger *et al.*, 1999; Wiegers *et al.*, 1999).

Recently, evidence for an alternative view of the role of CypA in the HIV-1 life cycle has emerged. Anti-CypA antibodies were found to block HIV-1 infection by preventing viral uptake (Sherry *et al.*, 1998). This result suggests that CypA acts at a very early step of HIV-1 infection—viral entry. However, to date, HIV-1 entry is thought to be mediated exclusively by gp120. In the present study, we attempt to reconcile the respective roles of CypA and gp120 in HIV-1 entry.

HIV-1 entry consists of distinct sequential steps: attachment of the particle to the surface of the target cells, fusion between viral and cell membranes, and ultimately, internalization of the viral genome into target cells. We developed specific viral attachment and internalization assays in order to delineate the individual roles of CypA and gp120 in these early steps of infection. First, we found that CypA-deficient viruses do not replicate because they fail to attach to target cells. We showed that anti-CypA antibodies block HIV-1 infection by preventing viral attachment. This led us to postulate that CypA plays a direct role in the attachment of the virus to target cells. Using a variety of approaches, we find that CypA, by interacting with cell surface heparans, governs the initial HIV-1 attachment. Surprisingly, these experiments seem

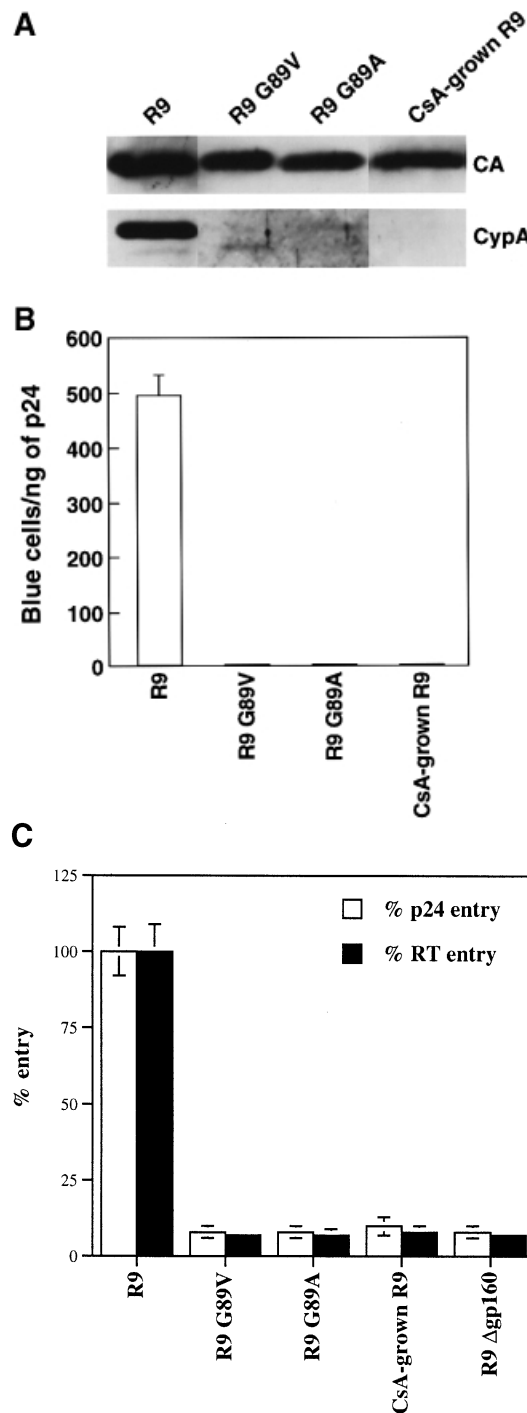
to preclude a role for gp120 in this initial step of the HIV-1 attachment process. Instead, several lines of evidence suggest that gp120 is crucial for the following steps of entry. We found that gp120 plays dual roles after the initial CypA-mediated attachment. First, gp120 stabilizes the adsorbed particles to the plasma membrane via CD4. Secondly, it induces rapid viral internalization within target cells. Thus, the present work reveals that incorporated host CypA and gp120 play distinct, but essential, roles for the initial step of HIV-1 infection—viral attachment.

## Results

### *CypA*-deficient viruses fail to enter target cells

The previous observation that anti-CypA antibodies block wild-type HIV-1 uptake (Sherry *et al.*, 1998) suggested that CypA participates in viral entry. Thus, we asked whether viruses that lack CypA fail to enter target cells. First, we generated mutant viruses that fail to incorporate CypA, but that are indistinguishable from wild-type viruses. Indeed, these mutant viruses exhibit wild-type biochemical and electron microscopic features (Grättinger *et al.*, 1999; Wiegers *et al.*, 1999). We introduced mutations in the CypA binding site of CA within the R9 proviral clone (NL-4.3) (Gallay *et al.*, 1997). Specifically, Gly89 was substituted by valine or alanine creating R9 G89V and R9 G89A clones, respectively. Consistent with previous studies (Grättinger *et al.*, 1999; Wiegers *et al.*, 1999), all of the mutations affected the efficiency of CypA incorporation (Figure 1A). Immunoblot analysis demonstrated that R9 G89V and R9 G89A mutant particles exhibited significantly diminished levels of CypA incorporation compared with the wild-type virus. Furthermore, in order to generate CypA-deficient viruses without genetic manipulation within the viral genome, CsA, an inhibitor of CypA-Gag complex formation, was added to the virus producer cells. These CsA-grown viruses also fail to incorporate CypA (Figure 1A), but otherwise are identical to wild-type particles as described previously (Thali *et al.*, 1994). We then measured the infectivity of the mutant viruses using adherent CD4+HeLa reporter cells. R9 G89V, R9 G89A and CsA-grown R9 that failed to incorporate CypA displayed a dramatic decrease in infectivity compared with the wild-type virus (Figure 1B).

After verifying that our CypA-deficient mutants are non-infectious, we tested their capacity to enter target cells. First, we compared the efficiency of entry between wild-type (R9) and CypA-deficient virus (R9 G89V or R9 G89A) by monitoring the presence of CA and reverse transcriptase (RT) within target cells. Adherent CD4+HeLa cells were infected with an identical amount of wild-type (R9) or CypA-deficient virus (R9 G89V or R9 G89A) (standardized by p24 or RT amounts) for 2 h at 37°C, washed and trypsin-treated to remove non-internalized material. It is important to note that *de novo* viral protein synthesis does not occur during this short time period (data not shown). Therefore, the only viral proteins detected in target cell lysates arise from the initial viral inoculum. We found that CypA-deficient viral entry was 10-fold less than wild-type entry (Figure 1C). To exclude the possibility that this defect is due to mutation within CA rather than to the inability to incorporate CypA,



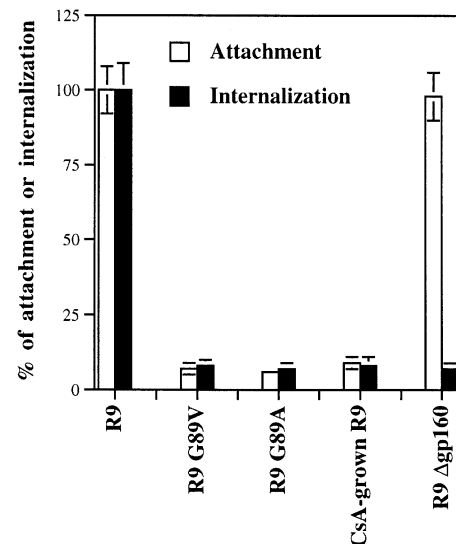
**Fig. 1.** Correlation between CypA incorporation and virus entry. (A) Purified wild-type and mutant viruses (3  $\mu$ g of p24 antigen per lane) from 293T transfected cells were analyzed for their CA and CypA content by immunoblot analysis. CsA (10  $\mu$ M) was added to 293T transfected cells 24 h post-transfection. (B) Purified viruses (1 ng of p24) were added to adherent CD4+HeLa cells. Infection was monitored by X-Gal staining. Results are expressed in number of blue cells per ng of p24. (C) Adherent CD4+HeLa cells were challenged with wild-type (R9), CypA-deficient (R9 G89V, R9 G89A or CsA-grown R9) or gp120-deficient (R9  $\Delta$ gp160) viruses for 2 h at 37°C. Viral inoculum was standardized by p24 antigen by ELISA (10 ng of p24). Target cells were washed to remove unbound virus and subsequently trypsinized to remove uninternalized virus. Entry levels were quantified by the measure of p24 or RT in cell lysates by p24 ELISA or an exoRT assay, respectively. Results are expressed as a percentage of p24 or RT entry by fixing the percentage of entry of wild-type virus at 100.

we performed the same experiments using CsA-grown R9 viruses and observed an identical failure for entry (Figure 1C). As expected, viruses that lack gp120 (R9  $\Delta$ gp160) also fail to enter target cells. This indicates that both CypA and gp120 are necessary for HIV-1 entry. It is important to emphasize that wild-type and mutant viruses used in the present study were all produced from 293T cells, harvested at the same time and purified under the same conditions. Therefore, potential vesicular contaminants containing viral proteins and that may be co-purified with viruses during ultracentrifugation are equally present in all virus preparations. Furthermore, it is crucial to note that the mutations introduced in the HIV-1 CA as well as the CsA-addition to virus producer cells do not affect either the release or the maturation of particles as described previously (Grättinger *et al.*, 1999; Wiegers *et al.*, 1999) and, thus, can not account for the inability of CypA-deficient viruses to enter into target cells. Taken together, these data demonstrate that the replicative defect of CypA-deficient viruses correlates with their failure to enter target cells.

#### **CypA is essential for the initial HIV-1 attachment to target cells**

Viral entry consists of distinct sequential steps: attachment of the particle to the surface of the target cells, fusion between viral and cell membranes, and ultimately, internalization of the viral genome into target cells. We developed viral attachment and internalization assays to localize more precisely the block for entry of CypA-deficient viruses, and to determine the respective roles of CypA and gp120 in this process. Comparison of different target cell lines for the attachment assay revealed that HIV-1 attachment to adherent cells (such as CD4+HeLa cells) is higher (a 10-fold increase) than to suspension cells (such as T lymphocytes). Given that adherent cells such as macrophages are a major target for HIV-1 infection, adherent cells were preferred to suspension cells to investigate the role of CypA in HIV-1 entry. To examine attachment exclusively, we first characterized the conditions that arrest particles at the extracellular side of the plasma membrane and yet exclude subsequent particle internalization events. These conditions were achieved by pre-cooling the target cells to 4°C for at least 1 h before the administration of the virus. Adherent CD4+HeLa cells, pre-cooled as above, were exposed for 30 min at 4°C to wild-type virus (R9), CypA-deficient viruses (R9 G89V, R9 G89A or CsA-grown R9), or gp120-deficient virus (R9  $\Delta$ gp160). Cells were then washed to remove unbound material and lysed. Viral attachment was monitored by measuring the amount of CA or RT in cell lysates. A dramatic decrease of attachment (~90%) was observed for CypA-deficient viruses (R9 G89V, R9 G89A or CsA-grown R9) compared with wild-type virus (Figure 2), suggesting that CypA plays a crucial role in the attachment of the virus to the plasma membrane of target cells. Surprisingly, no diminishment for attachment was measured for the gp120-deficient virus (R9  $\Delta$ gp160), suggesting that gp120 does not play a major role in the adsorption of the virus to adherent CD4+HeLa cells.

To explore the respective roles of CypA and gp120 in HIV internalization, cells pre-cooled for 1 h were exposed to virus for 30 min at 4°C, washed to remove unattached



**Fig. 2.** CypA incorporation is necessary for HIV-1 attachment to target cells. Purified wild-type (R9), CypA-deficient (R9 G89V, R9 G89A, CsA-grown R9) or gp120-deficient (R9  $\Delta$ gp160) viruses produced from 293T cells were tested for attachment or internalization on adherent CD4+HeLa cells. Attachment and internalization levels were quantified by the measure of p24 in cell lysates by p24 ELISA. Values are expressed as a percentage of attachment or internalization by fixing the percentage of attachment or internalization of wild-type virus at 100.

material and further incubated at 37°C to allow the pre-attached virus to be internalized. Target cells were then trypsinized to remove uninternalized viruses and lysed. We found that internalization of CypA-deficient viruses was 90% less than wild-type viruses (Figure 2), resulting from the defect for viral attachment observed above. Importantly, the gp120-deficient virus exhibited a dramatic defect for internalization (~90%) compared with the wild-type virus, confirming that gp120 is essential for viral internalization (Dagleish *et al.*, 1984). Similar results were obtained by monitoring RT (data not shown).

Our observations suggest that CypA plays a crucial role in HIV-1 attachment. Previous studies demonstrated that CypA-deficient viruses deleted for gp120, but pseudotyped with other viral envelopes, fail to infect target cells (Braaten *et al.*, 1996; Aiken, 1997). We asked whether this loss of infectivity arises from their failure to attach despite the presence of alternative viral envelope proteins. The amphotropic murine leukemia virus (MLV) or the vesicular stomatitis virus (VSV) envelopes, which both utilize CD4-independent entry pathways to infect target cells (Naldini *et al.*, 1996; Aiken, 1997), were used to pseudotype HIV-1 deleted for gp120 (R9  $\Delta$ gp160). Pseudotyped viruses were tested for both infectivity and attachment. Both MLV and VSV envelopes rescued the infectivity of R9  $\Delta$ gp160 (Table I) confirming that these proteins may substitute for gp120 in HIV-1 infection as described previously (Naldini *et al.*, 1996). In contrast, CypA-deficient virus deleted for gp120 (R9 G89V  $\Delta$ gp160), but pseudotyped with either MLV or VSV envelope protein, failed to be rescued for infectivity, consistent with previous findings (Braaten *et al.*, 1996; Aiken, 1997). Indeed, CypA-deficient viruses pseudotyped with either HIV-1, MLV or VSV envelope displayed levels of infectivity 100- to 5000-fold lower than pseudotyped

**Table I.** Pseudotyping with different viral envelopes does not rescue CypA-deficient HIV-1 infectivity or attachment

Infection <sup>a</sup>		Pseudotype			
	–	HIV-1 gp160	MLV Env	VSV Env	
R9 Δgp160	0	482 ± 37	187 ± 16	5645 ± 122	
R9 G89V Δgp160	0	0	0	0	
Attachment <sup>b</sup>		Pseudotype			
	–	HIV-1 gp160	MLV Env	VSV Env	
R9 Δgp160	246 ± 27	234 ± 25	241 ± 23	312 ± 34	
R9 G89V Δgp160	25 ± 2	26 ± 3	19 ± 2	34 ± 4	

<sup>a</sup>Purified viruses from transfected 293T cells were added to adherent CD4+HeLa indicator cells. Infection was monitored by X-Gal staining. Results are expressed as the number of blue cells per ng of p24.

<sup>b</sup>Purified viruses (10 ng of p24) from transfected 293T cells were added to adherent CD4+HeLa cells for 30 min at 4°C. Adherent cells were then washed to remove unbound material and lysed. Attachment levels were quantified by the measure of p24 in cell lysates by p24 ELISA. Results are expressed in pg of p24 (total captured amount).

wild-type viruses. Most importantly, pseudotyping CypA-deficient viruses (R9 G89V Δgp160) with either MLV or VSV envelope did not restore the capacity to attach to target cells (Table I), which was still 10-fold decreased compared with pseudotyped wild-type viruses. These results demonstrate that HIV-1 requires virus-associated proteins other than the viral envelope (gp120, MLV or VSV envelope) to adsorb to adherent target cells. Furthermore, these data emphasize the requirement of host CypA for the initial attachment of HIV-1 to target cells.

We showed that CypA is essential for the initial attachment of HIV-1 to target cells. Based on this observation, we postulated that CypA, directly or indirectly, is necessary for the initial adsorption of viral particles to the surface of target cells, allowing the subsequent interaction between gp120 with CD4 and coreceptors. To explore this hypothesis, we asked whether conditions that would obviate the need for the initial viral attachment, such as bringing CypA-deficient viruses into close contact with target cells, would rescue their infectivity. We speculated that placing CypA-deficient budding particles from transfected cells in proximity to the plasma membrane of target cells to allow the interaction between gp120 and CD4 would rescue their infectivity in the absence of CypA. To explore this issue, 293T cells transfected with wild-type virus (R9), CypA-deficient (R9 G89V), gp120-deficient (R9 Δgp160) or double CypA/gp120-deficient mutant virus (R9 G89V Δgp160) were used to infect adherent CD4+HeLa cells. Viral load was standardized by measuring the amount of CA in lysates of transfected cells. Microscopic analysis revealed that transfected cells came into tight contact via gravity with target cells (data not shown). In sharp contrast to cell-free viral infection (Figure 1B), cells transfected with CypA-deficient virus (R9 G89V) displayed levels of infectivity similar to those of wild-type virus-transfected cells (R9) (Table II) suggesting that cell-to-cell infection does not require CypA. As expected, cells transfected with gp120-deficient virus (R9 Δgp160 or R9 G89V

**Table II.** No requirement for CypA in HIV-1 cell-to-cell infection

	No. of blue cells per ng of p24
R9	4954 ± 38
R9 G89V	5031 ± 42
R9 Δgp160	0
R9 G89V Δgp160	0

293T cells transfected with R9, R9G89V, R9 Δgp160 or R9 G89V Δgp160 proviral DNA constructs (48 h post-transfection) were washed extensively to remove free viruses and used as inoculum to infect adherent CD4+HeLa cells. Cell-to-cell infection was subsequently processed as cell-free infection. Cellular inoculum was standardized by p24 antigen in cell lysate of 293T transfected cells. Results are expressed as the number of blue cells per ng of p24.

Δgp160) did not infect target cells. Taken together, these results reinforce the concept that CypA is essential for (cell-free particle) HIV-1 attachment and does not act in subsequent steps of infection such as viral uncoating or reverse transcription.

### Exposed CypA governs HIV-1 attachment

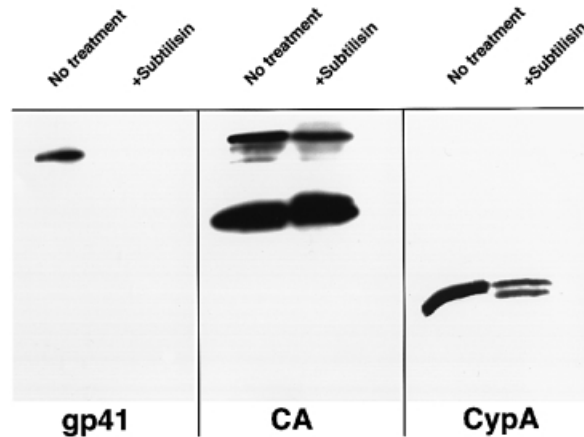
We demonstrated that the incorporation of host CypA is required for HIV-1 attachment. However, CypA may directly or indirectly participate in this process. In order to mediate HIV-1 attachment to target cells directly, one would expect that CypA is exposed at the surface of the viral membrane. To explore this possibility, we developed a virus-capture assay using anti-CypA antibodies. Wild-type, CypA-deficient (R9 G89V), gp120-deficient (R9 Δgp160) or CypA/gp120-deficient (R9 G89V Δgp160) viruses were added to plates coated with control (pre-immune), anti-matrix (MA), anti-CypA or anti-gp120 IgG (b12 IgG) (Burton *et al.*, 1991). Captured virus was quantified by p24 ELISA (Table III). Both anti-CypA and anti-gp120 IgG strongly bound wild-type virus (10- and 20-fold increases, respectively) compared with control and anti-MA IgG. Anti-gp120 IgG did not capture gp120-deficient viruses (R9 Δgp160 or R9 G89V Δgp160), demonstrating the specificity of the assay. Importantly, anti-gp120 captured R9 G89V virus similarly to wild-type virus indicating that CypA-deficient virus expresses wild-type levels of gp120 proteins at its surface. Furthermore, anti-CypA IgG did not capture CypA-deficient virus confirming the specificity of the assay. This result indicates that CypA molecules which may be present in the medium do not stick to the viral membrane, and may not explain the capture of wild-type virus by anti-CypA IgG. Double-deficient virus (R9 G89V Δgp160) did not bind to both anti-gp120 and anti-CypA IgG. Furthermore, pre-incubation of adsorbed anti-CypA IgG with an excess of exogenous recombinant CypA (300 μg) strongly decreased the capture of wild-type virus (Table III), further confirming the specificity of the assay. Altogether, these results suggest that incorporated CypA is exposed at the viral surface and therefore may act as a direct mediator of viral attachment.

Our observation that CypA is accessible to antibodies seems to contradict a previous study which shows that CypA remains protected from proteolytic treatment of intact viruses (Ott *et al.*, 1995). In order to reconcile these data with our own observation, we postulated that only a

**Table III.** Anti-CypA IgG captures HIV-1 in a CypA-dependent manner

Adsorbed IgG	R9	R9 G89V	R9 Δgp160	R9 G89V Δgp160
Control	26 ± 4	21 ± 2	31 ± 5	28 ± 3
Anti-MA	29 ± 3	27 ± 4	26 ± 3	28 ± 4
Anti-gp120 (b12)	424 ± 37	292 ± 44	36 ± 5	29 ± 3
Anti-CypA	394 ± 31	27 ± 3	408 ± 33	31 ± 3
Anti-CypA + excess rec. CypA	48 ± 5	26 ± 4	41 ± 3	29 ± 3

Purified viruses (10 ng of p24) from 293T transfected cells were incubated for 2 h at 37°C with the adsorbed IgG. Plates were washed and bound viruses were lysed. Results are expressed in pg of p24 (total captured amount). An excess of recombinant (rec.) human CypA (300 µg) was added together with the virus to adsorbed anti-CypA IgG.



**Fig. 3.** Incorporated CypA is susceptible to proteolytic digestion in intact particles. Purified wild-type virus (R9) from 293T transfected cells was exposed to subtilisin and analyzed for viral component content by immunoblot analysis (20% acrylamide-SDS gel) using anti-gp41, anti-CA and anti-CypA IgG.

small portion of CypA is exposed to the extraviral medium. In this instance, only a small part of CypA would be removed upon proteolytic cleavage, and this smaller species could only be resolved by using high resolution PAGE. Because this previous study could not resolve a difference in size between the 18 and 19 kDa CypA isoforms (Ott *et al.*, 1995), their immunoblot analysis of protease-treated viruses may not have been able to distinguish between intact CypA and a proteolytically truncated form. To explore this possibility, purified wild-type viruses (R9) from 293T transfected cells were treated with subtilisin and analyzed by immunoblotting (20% acrylamide-SDS gel) for the presence or absence of CypA. It is important to emphasize that the same blot has been successively probed with anti-gp41, anti-CA and anti-CypA IgG. The surface protein gp41 was digested, but internal proteins, Gag (p55) and CA (p24), were not digested (Figure 3). The majority of CypA molecules were partially digested upon protease treatment, indicating that a part of incorporated host CypA is exposed at the viral surface. Note that only one isoform of CypA is detected within R9 viruses produced from 293T transfected cells. Importantly, we found that CypA, along with known surface exposed proteins (gp41 and gp120), is accessible to biotinylation, whereas internal proteins (CA and MA) are not (data not shown).

A previous study showed that anti-CypA antibodies inhibit HIV-1 infection (Sherry *et al.*, 1998). As our results above demonstrate that CypA is exposed at the surface of the virus, we next sought to determine whether these anti-

**Table IV.** Anti-CypA IgG inhibits HIV-1 infection by blocking attachment to target cells

Antibodies	Attachment <sup>a</sup>	Infection <sup>b</sup>
–	100	100
Control	97 ± 7	97 ± 11
Anti-MA	95 ± 8	95 ± 12
Anti-gp120 (15e)	97 ± 11	0 ± 0
Anti-CypA	9 ± 3	11 ± 4

<sup>a</sup>Purified wild-type virus (R9) pre-incubated for 1 h at 37°C with control, anti-MA, anti-gp120 (15e) or anti-CypA IgG (20 µg/ml final concentration) was tested for its capacity to attach adherent CD4+HeLa cells as above (see Table I). Results of attachment are expressed as a percentage of attachment by fixing wild-type adsorption in the absence of antibody at 100.

<sup>b</sup>Purified viruses (1 ng of p24) from transfected 293T cells were added to adherent CD4+HeLa cells. Control, anti-MA, anti-gp120 (15e) or anti-CypA IgG (20 µg/ml final concentration) were added together with the virus to target cells. Infection was monitored by X-Gal staining. Results of infectivity are expressed as a percentage of infection by fixing wild-type infection in the absence of antibody at 100.

CypA antibodies disrupt the viral attachment process. First, we verified the inhibitory effect of anti-CypA IgG on HIV-1 infection. Adherent CD4+HeLa cells were infected with wild-type virus in the presence of control, anti-MA, anti-CypA or neutralizing anti-gp120 IgG (15e IgG) (Robinson *et al.*, 1990). Control IgG had no effect on wild-type infectivity, while anti-CypA IgG inhibited HIV-1 infection (Table IV), consistent with a previous study (Sherry *et al.*, 1998). As previously reported (Thali *et al.*, 1991), neutralizing anti-gp120 IgG suppressed viral infectivity. These results further confirm that both CypA and gp120 are key players in HIV-1 infection.

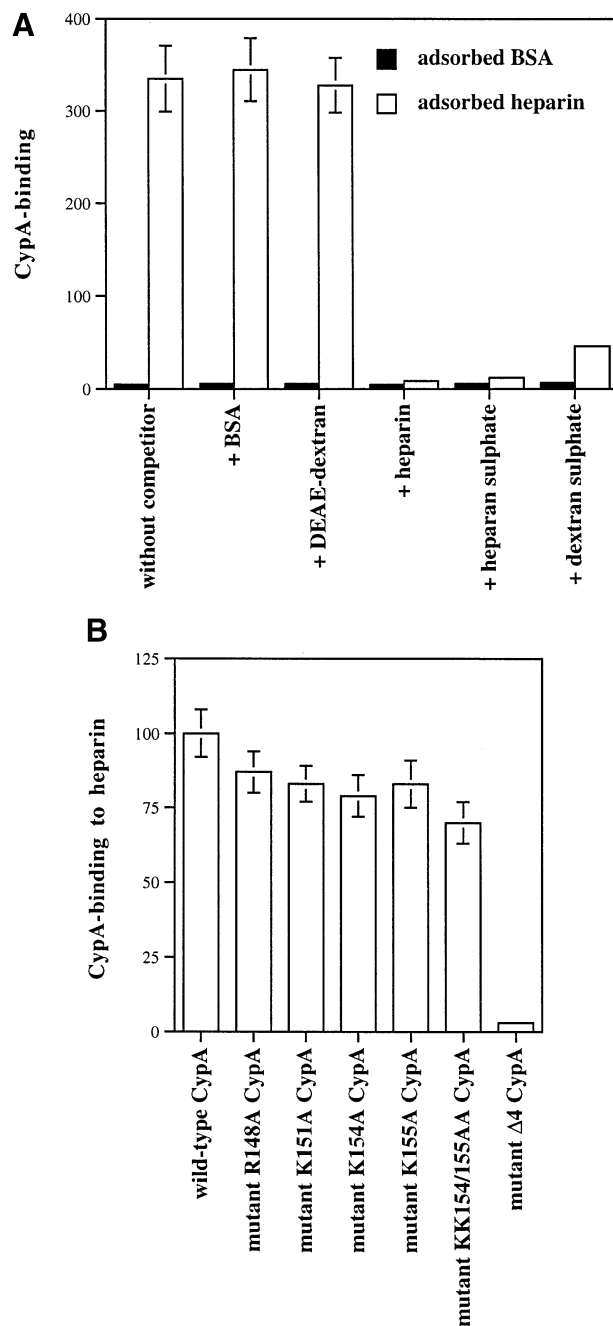
We next asked whether the anti-CypA antibodies inhibit HIV-1 infection by preventing the initial CypA-mediated viral attachment. A viral attachment assay was performed using wild-type virus (R9) in the presence of control, anti-CypA, anti-gp120 (15e IgG directed the CD4-binding site of gp120) or anti-MA IgG. Control, anti-MA and anti-gp120 IgG did not affect viral attachment (Table IV). In contrast, anti-CypA IgG prevented ~90% of the viral adsorption. This inhibitory effect is not due to non-specific adherence of CypA molecules originating from the extraviral medium to HIV-1, since CypA-deficient mutant viruses (R9 G89V) produced under the same conditions as wild-type viruses are not recognized by anti-CypA IgG (Table III). The inhibitory effect of anti-CypA IgG on viral adsorption suggests that CypA molecules exposed at the viral surface mediate HIV-1 attachment. These results explain the previous observation that anti-

CypA antibodies block HIV-1 infection (Sherry *et al.*, 1998). Note that anti-CypA antibodies do not affect the infectivity of viruses (HIV-2 Rod) that do not require CypA for replication (data not shown). Together these data confirm our previous observations that exposed CypA plays a key role in HIV-1 attachment, and map the precise step in HIV-1 infection where anti-CypA antibodies act. Furthermore, the direct correlation between CypA-mediated viral attachment and infectivity indicates that viral adsorption is a crucial and limiting step for HIV-1 replication.

#### CypA binds to target cells via heparans

Our observation that anti-CypA antibodies prevent HIV-1 attachment suggests that CypA participates directly in this process. This also suggests the existence of specific receptors at the surface of target cells for CypA. Supporting this hypothesis, a recent study revealed the presence of saturable CypA-binding sites on CD4+ T lymphocytes (Sherry *et al.*, 1998). Interestingly, a glycosaminoglycan protein has been identified as a candidate surface receptor (Denys *et al.*, 1998) for another member of the immunophilin family, cyclophilin B (CypB). Indeed, CypB was found to bind directly to heparin *in vitro* (Denys *et al.*, 1998). A glycosaminoglycan is a linear saccharide possessing a characteristic disaccharide sulfated repeat sequence (Jackson *et al.*, 1991). The most common glycosaminoglycan structures are heparan sulfate, chondroitin sulfate, dermatan sulfate and keratan sulfate. These glycosaminoglycan chains are covalently attached to a core protein; the resulting macromolecules are termed proteoglycans (Jackson *et al.*, 1991). Most importantly, a large number of studies have demonstrated that heparan sulfate plays a role in HIV-1 replication. Specifically, heparinase treatment, which removes heparans exclusively, blocks both attachment and infectivity of HIV-1 (Mondor *et al.*, 1998). Furthermore, addition of soluble heparans totally abolishes HIV-1 infection by preventing virus attachment (Mondor *et al.*, 1998). Based on these observations, we investigated the possibility that the CypA-binding site is a cell surface heparan. To explore this issue, we asked whether CypA binds directly to heparans. We developed an *in vitro* binding assay using heparin adsorbed onto a 96-well plate as a solid phase to capture CypA. Bound CypA was detected by ELISA using anti-CypA IgG. Importantly, we found that CypA binds to heparin, but not to bovine serum albumin (BSA) (Figure 4A). Furthermore, the addition of a variety of soluble heparan derivatives such as heparin, heparan sulfate or dextran sulfate prevents CypA-binding to adsorbed heparin, whereas BSA or uncharged DEAE-dextran do not inhibit the CypA-heparin interaction. These results suggest that CypA possesses the ability to bind directly and specifically to heparin, and implicate CypA as a potential direct mediator for HIV-1 attachment to target cells via heparans.

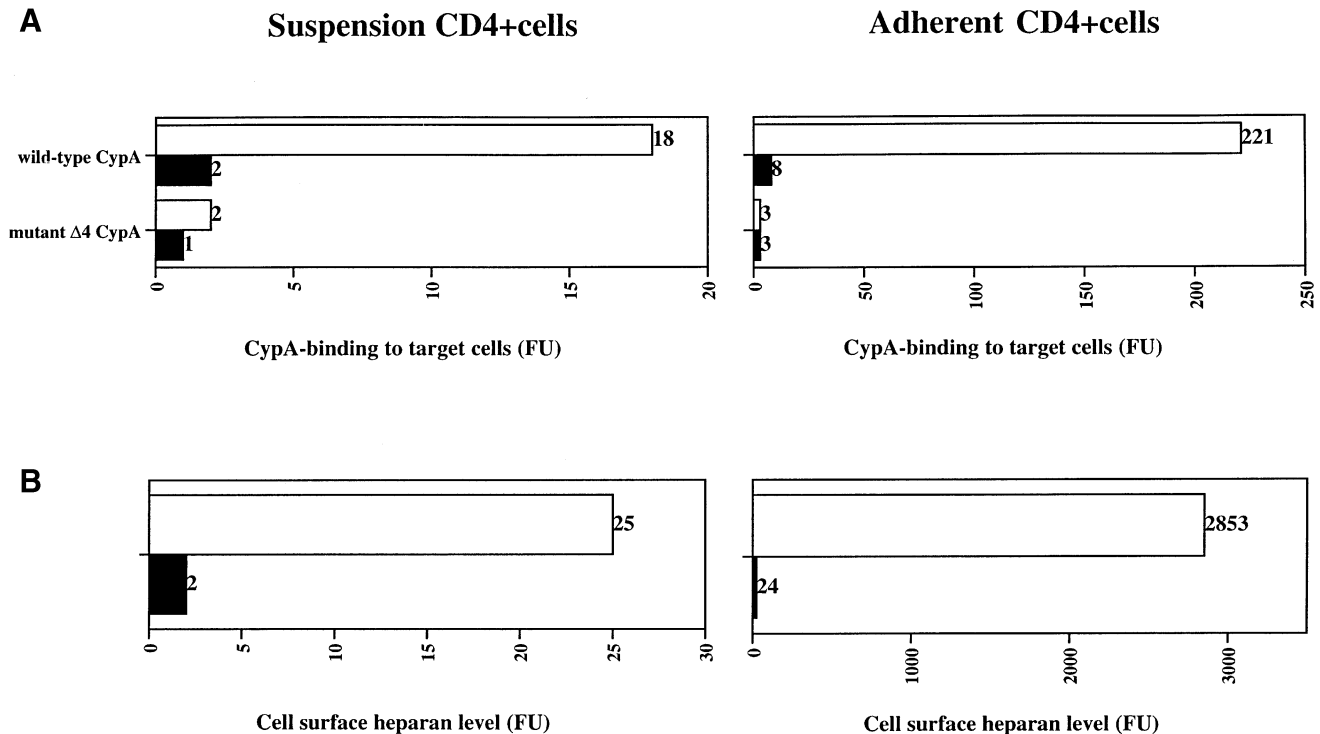
We next sought to identify the domains of CypA that are responsible for its interaction with heparans. Previous studies showed that the majority of heparin-binding proteins bind to heparin via a consensus sequence rich in basic residues (Hileman *et al.*, 1998). Interestingly, we found that the C-terminus of CypA contains such a region (<sup>148</sup>RNGKTSKK<sup>155</sup>). To determine whether this domain plays a role in CypA-binding to heparin, each basic residue



**Fig. 4.** CypA binds to heparin via its basic C-terminus.

(A) Recombinant CypA was added to adsorbed BSA or heparin in the presence or absence of competitors such as BSA, DEAE-dextran, heparin, heparan sulfate or dextran sulfate. CypA bound to adsorbed heparin was detected by ELISA using anti-CypA IgG. Values are expressed in units of absorbance. (B) Recombinant wild-type, mutant R148A, mutant K151A, mutant K154A, mutant K155A, double-mutant KK154/155AA or tetra-mutant RKKK148/151/154/155AAAA ( $\Delta 4$ ) CypA were incubated with adsorbed heparin. Bound CypA was detected as above. Results are expressed as a percentage of binding to adsorbed heparin by fixing wild-type CypA-binding at 100.

of this region was replaced by an alanine, and the resulting CypA mutants were tested for their capacity to bind to heparin. Single point mutations (R<sup>148</sup>A, K<sup>151</sup>A, K<sup>154</sup>A or K<sup>155</sup>A) or a double point mutation (KK<sup>154/155</sup>AA) decrease the binding of CypA to heparin slightly (Figure 4B). Importantly, a CypA mutant in which all four basic residues were substituted by alanine (termed  $\Delta 4$  mutant)



**Fig. 5.** CypA binds to target cells via heparans. **(A)** CypA-binding to target cells requires cell surface heparans. Suspension CD4+cells (H9) (left panels) or adherent (CD4+HeLa) (right panels), pretreated or not with heparinase, were incubated with recombinant wild-type or tetra-mutant CypA ( $\Delta 4$ ). Bound CypA was detected by FACS using anti-CypA IgG. Values are expressed in fluorescence units (FU). Results represent the summation of three independent experiments. **(B)** The high level of CypA-binding to adherent cells correlates with their elevated cell surface level of heparans. Levels of cell surface heparans on suspension or adherent cells were determined by FACS analysis using anti-heparan antibodies. Values are expressed in fluorescence units (FU). Results represent the summation of three independent experiments. (□) Without heparinase treatment; (■) with heparinase treatment.

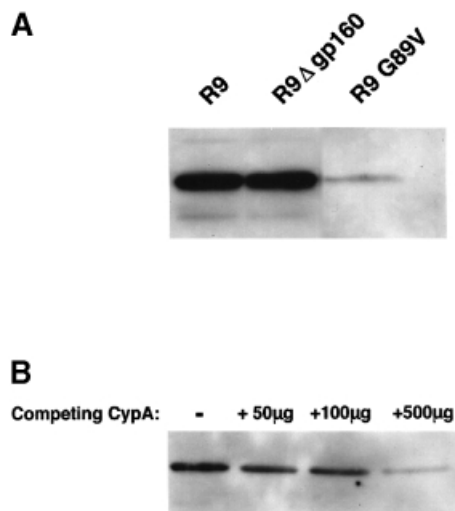
lost its capacity to bind heparin (Figure 4B). This result indicates that CypA binds to heparin via its basic C-terminus, and that all four basic residues are necessary for this interaction. Importantly, analysis of the crystal structure of CypA reveals that all four basic residues are exposed at the surface of the molecule (Gamble *et al.*, 1996). It is noteworthy that all of the CypA mutant proteins, including the  $\Delta 4$  mutant, bind to HIV-1 CA *in vitro* at levels similar to those of wild-type CypA (data not shown), suggesting that these mutant proteins are properly folded.

After identifying the region of CypA responsible for its interaction with heparin, we asked whether CypA binds to target cells via heparans. To explore this issue, wild-type or mutant CypA ( $\Delta 4$ ) were tested for their capacity to bind to CD4+adherent (CD4+HeLa cells) or CD4+suspension (H9 T lymphocytes) cells. Target cells were pretreated or not with heparinase that removes heparan sulfate moieties. Membrane-bound CypA was detected by FACS analysis using anti-CypA IgG. First, we found that CypA binding to adherent cells is greater than to suspension cells (10-fold more, 221FU versus 18FU), suggesting that adherent cells express higher levels of CypA-binding sites than suspension cells (Figure 5A). Importantly, wild-type CypA binds to target cells, whereas mutant CypA ( $\Delta 4$ ) does not. Furthermore, heparinase treatment totally abolished wild-type CypA-binding to target cells to levels similar to those of mutant CypA ( $\Delta 4$ ). These results suggest that CypA binds to target cells exclusively via heparans, and this interaction is

mediated via the basic C-terminus of CypA. Removal of heparans by enzymatic treatment was verified by FACS analysis using anti-heparan IgM (Figure 5B). Importantly, levels of heparans on adherent cells were 100-fold higher than those on suspension cells (2853FU versus 25FU). It is important to note that CypA binds to wild-type CHO cells, but fails to bind to glycosaminoglycan-deficient CHO cells (data not shown). This result reveals a direct correlation between the capacity of target cells to bind CypA and the levels of cell surface heparan expression. Altogether, these data demonstrate that CypA binds to target cells exclusively via heparans.

#### **HIV-1 requires CypA-heparan interactions to attach**

Our work demonstrates that CypA possesses the capacity to bind to target cells via heparans. Thus, we asked whether CypA directly mediates HIV-1 attachment to target cells via heparans. First, we investigated the ability of HIV-1 to bind heparans *in vitro*. Wild-type (R9), gp120-deficient (R9  $\Delta$ gp160) or CypA-deficient (R9 G89V) viruses were tested for their ability to bind to heparin-coupled beads or BSA-coupled beads. Bound viruses were eluted and analyzed by immunoblotting using anti-CA IgG. Importantly, both wild-type and gp120-deficient viruses bind to heparin, whereas CypA-deficient viruses do not (Figure 6A). None of the viruses bind to BSA-beads (data not shown). This result suggests that CypA, but not gp120, is necessary for HIV-1 binding to heparin. To demonstrate further that CypA mediates HIV-1 attach-



**Fig. 6.** HIV-1 binds to heparin via CypA. (A) Purified R9, R9  $\Delta$ gp160 or R9 G89V viruses from transfected 293T cells were incubated with heparin beads. Bound virus was detected by immunoblot analysis using anti-CA IgG. (B) Purified wild-type virus (R9) was incubated with heparin beads in the presence of increasing concentrations of recombinant CypA (50, 100, 500  $\mu$ g). Bound virus was detected by immunoblot analysis using anti-CA IgG.

ment to heparin, wild-type viruses were tested for their ability to bind to heparin beads in the presence of an excess of exogenous recombinant wild-type CypA. Importantly, exogenous wild-type CypA, by saturating all heparin-binding sites, strongly decreased HIV-1 capture by heparin beads (Figure 6B). These data strongly suggest that CypA is necessary for HIV-1 binding to heparans.

After demonstrating that CypA is required for HIV-1 binding to heparans, we investigated whether HIV-1 attaches to target cells via the CypA–heparan interaction. First, we verified the requirement of cell surface heparans for HIV-1 attachment. Wild-type (R9), CypA-deficient (R9 G89V) or gp120-deficient (R9  $\Delta$ gp160) viruses were tested for their ability to attach to suspension or adherent CD4<sup>+</sup> cells, pretreated or not with heparinase. Interestingly, levels of HIV-1 attachment to adherent cells were 8-fold increased compared with those of suspension cells (476 versus 60 pg of p24) (Figure 7). Given that adherent cells expressed higher levels of heparans than suspension cells (Figure 5B), this supports the notion that HIV-1 attaches to target cells via heparans. Most importantly, both wild-type and gp120-deficient viruses fail to attach to heparinase-treated target cells (suspension or adherent cells), demonstrating the strict requirement for heparans in HIV-1 attachment. Note that HIV-1 attaches to wild-type CHO cells, but fails to attach to glycosaminoglycan-deficient CHO cells (data not shown). To demonstrate that HIV-1 attaches to cell surface heparans via incorporated CypA, target cells were pre-incubated with an excess of exogenous recombinant wild-type or mutant ( $\Delta$ 4) CypA to saturate their cell surface heparans, and then tested for their ability to bind wild-type (R9), CypA-deficient (R9 G89V) or gp120-deficient (R9  $\Delta$ gp160) viruses. Importantly, saturation of cell surface heparans with exogenous wild-type CypA totally abolished HIV-1 attachment (Figure 7), whereas mutant  $\Delta$ 4 CypA did not affect viral adsorption. This confirms the previous observation that CypA binds to target cells in a saturable manner (Sherry

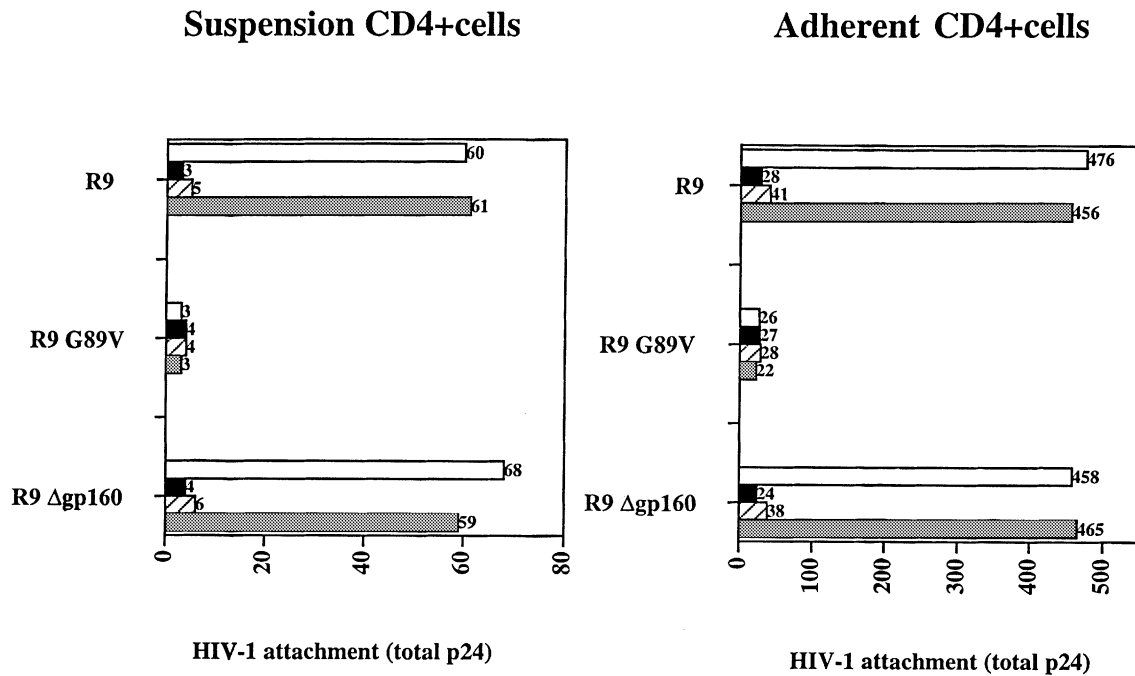
*et al.*, 1998). Altogether, these results strongly suggest that CypA exposed at the surface of the virus, by interacting with cell surface heparans, permits HIV-1 to attach to target cells.

#### **Initial CypA-mediated attachment is necessary for the subsequent gp120–CD4 interaction**

Our present work suggests that CypA, but not gp120, is necessary for HIV-1 to attach to target cells via heparans. This seems to contradict the prevailing view of gp120 as the main mediator for HIV-1 attachment. One important distinction between our study and the majority of these studies is that we used adherent cells, while others used suspension cells as targets for attachment assays. Our present work exhibits profound discrepancies between adherent and suspension cells. Levels of heparans on adherent cells are strikingly higher (100-fold) than those on suspension cells (Figure 5B). This elevated number of potential CypA-binding sites correlates with the high levels of CypA binding (Figure 5A) and the high levels of HIV-1 attachment to adherent cells (Figure 7) compared with those of suspension cells. These observations suggested to us that the use of adherent or suspension cells for attachment studies may result in divergent interpretations in the respective roles of gp120 and CypA. Corroborating this hypothesis, Sattentau and co-workers, using an elegant system based on the detection of HLA-DR<sup>+</sup> virus attached to HLA-DR<sup>−</sup> target cells, found a profound discrepancy between HIV-1 attachment to suspension CD4<sup>+</sup> cells (T lymphocytes) and adherent CD4<sup>+</sup> cells (CD4<sup>+</sup>HeLa cells) (Mondor *et al.*, 1998). Specifically, they showed that the majority of neutralizing anti-gp120 antibodies directed against the CD4-binding site of gp120 prevent viral attachment to suspension CD4<sup>+</sup> cells, but do not inhibit attachment to adherent CD4<sup>+</sup> cells (Mondor *et al.*, 1998). Furthermore, this study demonstrated that CD4 is required for HIV-1 attachment to suspension cells, but not to adherent cells (CD4<sup>+</sup>HeLa cells) (Mondor *et al.*, 1998). These results together with ours confirmed that HIV-1 uses interactions other than gp120–CD4, such as CypA–heparan interactions, to attach to adherent cells. This also suggests that the use of adherent cells instead of suspension cells permits discrimination between gp120-dependent and gp120-independent events in the HIV-1 attachment process.

How can the fact that gp120 is found to be necessary for viral attachment to suspension, but not to adherent CD4<sup>+</sup> cells, be reconciled with our observations that gp120 does not participate in viral attachment to either type of cell? Given that an attachment assay using suspension cells includes multiple washes by centrifugation and resuspensions of pelleted cells, and given that suspension cells exhibit very low levels of heparans (Figure 5B), we postulated that these mechanical procedures release viruses that are attached only via CypA–heparan interactions, but not those that are stabilized by subsequent gp120–CD4 interactions. Thus, viruses stabilized by gp120–CD4 interactions will be the predominant population of attached viruses detectable on suspension cells. This hypothesis would explain the previously described requirement of gp120 in HIV-1 attachment to suspension cells. To explore this hypothesis, we assayed washing conditions that preserve the virus attached to target cells exclusively via



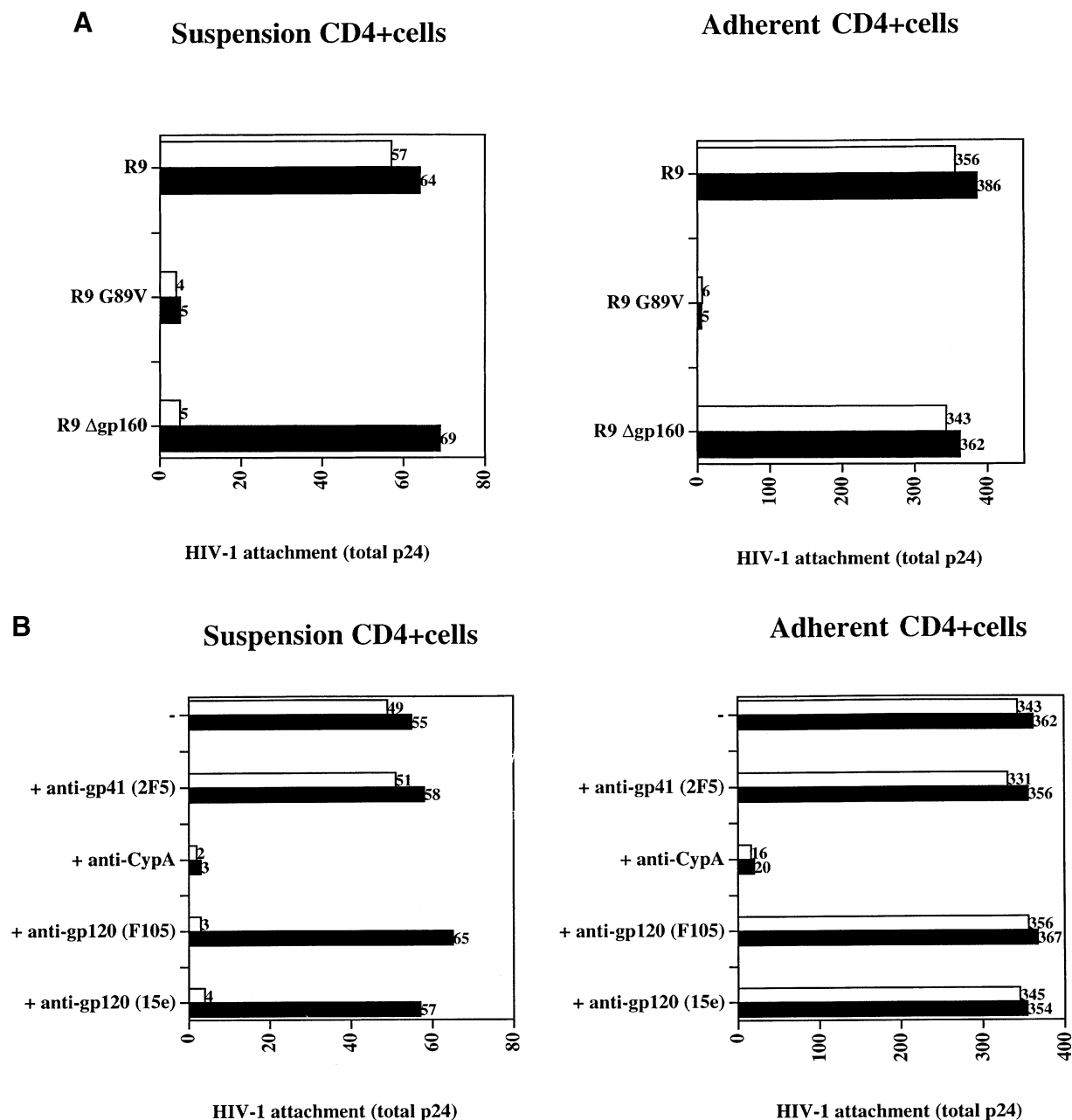


**Fig. 7.** The basic C-terminus of CypA mediates HIV-1 attachment to target cells via heparans. Suspension CD4+ cells (H9) (left) or adherent (CD4+HeLa) (right) treated or not with heparinase were tested for their capacity to attach HIV-1 in the presence or absence of an excess of exogenous wild-type or tetra-mutant ( $\Delta 4$ ) CypA (500  $\mu$ g). Purified R9, R9 G89V or R9  $\Delta$ gp160 viruses (10 ng of p24) from 293T transfected cells were incubated with target cells for 30 min at 4°C. The amount of attached virus was quantified by p24 ELISA. Values are expressed in pg of p24 (total attached amount). (□) No treatment; (■) with heparinase treatment; (▨) + excess wild-type CypA; (○) + excess mutant  $\Delta 4$  CypA.

CypA. Interestingly, we found that ‘standard’ washing techniques, which employ a washing buffer stream directly onto pelleted target cells, cause the release of viruses attached to suspension cells exclusively via CypA. In sharp contrast, ‘gentle’ washing procedures that avoid the use of a direct buffer stream onto target cells maintain virus attached exclusively via CypA. It is important to note that only the ‘gentle’ washing procedure was used in all the above attachment assays. Using ‘gentle’ washing procedures, wild-type (R9) and gp120-deficient (R9  $\Delta$ gp160) viruses attach similarly to suspension or adherent cells, whereas CypA-deficient viruses fail to attach (Figure 8A). This result further confirms that CypA, but not gp120, is necessary for HIV-1 attachment to both types of target cells. The fact that CypA-deficient viruses fail to attach to target cells demonstrates that ‘gentle’ washing conditions are sufficient to remove unattached virus. As previously described (Figure 7), levels of attachment to suspension cells were 8-fold decreased compared with those of adherent cells. Importantly, a completely different pattern of viral attachment was observed using ‘standard’ washing procedures. Indeed, gp120-deficient viruses fail to attach to suspension cells, whereas wild-type viruses do attach. These results suggest that gp120-deficient viruses remain attached to suspension cells under ‘gentle’ washing procedures, but are released upon ‘standard’ washing procedures. This corroborates our hypothesis that gp120 is not necessary for the initial CypA-mediated attachment, but is crucial to subsequently stabilize attached viruses during mechanical agitation. Importantly, viruses deleted for gp120 remain attached to adherent cells even using ‘standard’ washing procedures, suggesting that the avidity of adherent cells for HIV-1 is greater than that of suspension cells. Given that cell

surface levels of heparans on adherent cells are higher than those of suspension cells (Figure 5B), it is likely that the higher number of CypA–heparan interactions is sufficient to retain gp120-deleted viruses attached at the cell membrane despite mechanical agitation. These results corroborate our hypothesis that CypA is necessary for the initial viral attachment, whereas gp120 is essential to subsequently stabilize the adsorbed particles at the surface of target cells.

We next asked whether gp120 stabilizes attached HIV-1 via CD4. We tested the effect of anti-gp120 IgG directed against the CD4-binding site of gp120 (15e and F105 IgG) (Robinson *et al.*, 1990; Posner *et al.*, 1991) on the stability of attached HIV-1 to target cells. An antibody directed against gp41 (2F5) (Buchacher *et al.*, 1992) was used as a negative control, whereas anti-CypA IgG was used as a positive control. Wild-type viruses pre-incubated with the above antibodies were tested for attachment to suspension and adherent cells. We examined the effect of ‘gentle’ or ‘standard’ washing of target cells on HIV-1 attachment in the presence of the above antibodies. Using ‘gentle’ washing procedures, anti-gp120 or anti-gp41 IgG do not affect HIV-1 attachment to both suspension and adherent cells (Figure 8B), confirming that the gp120–CD4 interaction does not participate in the initial viral attachment. In contrast, using ‘standard’ washing procedures, antibodies directed against the CD4-binding site of gp120 (F105 and 15e) totally abrogate HIV-1 attachment to suspension cells, whereas they do not prevent viral attachment to adherent cells. These results confirm a previous study which shows that neutralizing anti-gp120 IgG directed against the CD4-binding site of gp120 prevents HIV-1 attachment to suspension CD4+ cells, but does not prevent attachment to adherent CD4+ cells



**Fig. 8.** Comparison between HIV-1 attachment to CD4+suspension or CD4+adherent cells permits the discrimination between the CypA-heparan interaction and the gp120-CD4 interaction. (A) Suspension CD4+cells (H9) (left) or adherent (CD4+HeLa) (right) were incubated with purified R9, R9 G89V or R9 Δgp160 (10 ng of p24). Target cells were washed using either a 'standard' (□) or 'gentle' (■) procedure (see Materials and methods). Attached virus was quantified by p24 ELISA. Values are expressed in pg of p24 (total attached amount). (B) Viral attachment to suspension or adherent cells was performed as above in the presence of anti-gp41 (2F5), anti-CypA, anti-gp120 IgG directed against the CD4-binding site of gp120 (F105 and 15e) (20 μg/ml). Values are expressed in pg of p24 (total attached amount).

(Mondor *et al.*, 1998). These observations together with ours strongly suggest that gp120 stabilizes attached viruses via CD4 after the initial CypA-mediated attachment. It is important to emphasize that anti-CypA IgG prevents HIV-1 attachment to both suspension or adherent cells using 'gentle' or 'standard' cell washing, further demonstrating the strict requirement of CypA for the initial step of HIV-1 attachment. Most importantly, these results indicate that the use of suspension or adherent cells, as well as the conditions of resuspension of target cells, are

critical parameters in the characterization of the respective roles of CypA and gp120 in HIV-1 attachment.

## Discussion

The experiments presented in this report shed light on the mechanism of action of CypA in the HIV-1 life cycle. Based on our observations, we propose the following model. Host CypA is specifically incorporated into particles via an interaction with the Gag precursor. CypA

undergoes a subviral relocation to the surface of the virus. This CypA exposure permits the attachment of the virus to target cells via CypA–heparan interactions. This initial CypA-mediated virus adsorption allows the subsequent interaction between gp120 with CD4 and coreceptors leading to viral internalization.

### ***CypA, but not gp120, is required for the initial step of HIV-1 attachment***

Our experiments tracing the internalization of viral proteins such as CA and RT demonstrate that CypA incorporation into the virus is a prerequisite for efficient HIV-1 entry into target cells. This is in accordance with a previous study reporting that CypA is required in the infectious cycle prior to the initiation of reverse transcription (Braaten *et al.*, 1996). Here, we show that the block in viral entry of CypA-deficient virus occurs at the initial step of HIV-1 infection—viral attachment.

A variety of experimental evidence suggests that CypA is required for the initial viral attachment to target cells. First, we demonstrated that viruses which lack CypA fail to attach to target cells. Importantly, these viruses that fail to incorporate CypA are otherwise identical to wild-type particles as previously described (Thali *et al.*, 1994; Grättinger *et al.*, 1999; Wiegers *et al.*, 1999). It is noteworthy that this failure of CypA-deficient viruses to attach was observed on all types of human cells tested, such as HeLa cells, macrophages or peripheral blood lymphocytic cells (data not shown), suggesting that the requirement for CypA in HIV-1 attachment is ubiquitous. This is consistent with the fact that CypA-deficient viruses fail to replicate *in vitro* in peripheral blood mononuclear cells that include both T lymphocytes and macrophages—the two major virus reservoirs in HIV-1-infected individuals. Secondly, either anti-CypA antibodies or an excess of exogenous CypA prevent HIV-1 infection by blocking viral adsorption, demonstrating that CypA is a major determinant for the initial attachment of the virus to target cells.

Our work suggests that gp120 does not participate in the initial CypA-mediated attachment to target cells. First, adsorption levels of gp120-deficient or wild-type viruses to adherent cells are similar. Furthermore, neutralizing anti-gp120 IgG, which prevents the gp120–CD4 interaction, does not influence HIV-1 attachment to adherent cells. Importantly, the same results are observed for suspension cells using ‘gentle’ washing conditions. Altogether these data suggest that the gp120–CD4 interaction is not necessary for the initial step of attachment. These observations are consistent with a recent study which reports that HIV-1 attaches similarly to CD4-positive or -negative HeLa cells (Mondor *et al.*, 1998). This study also shows that anti-gp120 IgG directed against the CD4-binding site of gp120 does not affect HIV-1 attachment to CD4+HeLa cells, further demonstrating that HIV-1 attachment to target cells is CD4-independent (Mondor *et al.*, 1998). Secondly, our work demonstrates that MLV or VSV pseudotyping does not restore the adsorption of CypA-deficient virus, indicating that CypA is absolutely required for the initial HIV-1 attachment independent of the viral envelope. This agrees with previous studies showing that pseudotyping does not rescue CypA-deficient virus infectivity (Braaten *et al.*, 1996;

Aiken, 1997). Thirdly, although CypA-deficient viruses (CA mutants or CsA-grown viruses) express wild-type levels of gp120 protein, they do not attach to target cells. Fourthly, we show that the requirement of CypA for infectivity can be bypassed by using systems that obviate the need for the initial viral attachment such as cell-to-cell transmission. Fifthly, enzymatic removal of CypA-binding sites—the heparans—completely abrogates both wild-type or gp120-deficient viral attachment to suspension or adherent cells. Altogether, these results suggest an absolute requirement for CypA in the initial step of HIV-1 infection—viral attachment—and exclude the participation of gp120 in this initial attachment process.

### ***The CypA–heparan interaction precedes the gp120–CD4 interaction***

Our results suggesting that gp120 is not necessary for the initial step of HIV-1 attachment seem to contradict previous studies that demonstrate an absolute requirement of gp120 for viral adsorption. However, the present study using several approaches suggests an alternative role for gp120 in the HIV-1 attachment process that may reconcile this apparent paradox. Importantly, this study demonstrates that the use of suspension or adherent cells as targets for attachment assays, as well as the washing procedures used on target cells, are crucial determinants in the understanding of the respective roles of CypA and gp120 in HIV-1 attachment. Here, we present evidence that gp120 is essential to retain attached particles to the surface of target cells via CD4 after the initial CypA-mediated HIV-1 attachment.

We demonstrate that the conditions of resuspension of target cells in attachment assays are a critical parameter to delineate the precise role of gp120 in HIV-1 adsorption. We observe that ‘standard’ washing procedures permit detection of viruses that are stably attached to suspension cells via gp120–CD4 interactions, but do not allow detection of viruses that are attached exclusively via the CypA–heparan interaction. In contrast, ‘gentle’ washing of suspension cells permits detection of viruses that are attached either via gp120–CD4 or CypA–heparan interactions. Specifically, we show that wild-type or gp120-deficient viruses attach similarly to suspension cells using ‘gentle’ washing conditions, whereas CypA-deficient viruses do not attach. Furthermore, neutralizing anti-gp120 antibodies directed against the CD4-binding site of gp120 do not influence HIV-1 attachment to suspension cells under ‘gentle’ washing procedures. This further demonstrates that gp120 does not participate in the initial virus attachment. In sharp contrast, ‘standard’ washing of suspension cells induces the release of attached gp120-deficient viruses, whereas wild-type viruses remain attached. It is likely that under these artificial experimental conditions, the low number of CypA-binding sites (heparans) on suspension cells is not sufficient to maintain viruses attached exclusively via the CypA–heparan interaction, but not yet attached via the gp120–CD4 interaction. Importantly, we demonstrate that the gp120–CD4 interaction allows wild-type viruses to remain attached to target cells despite the harsh mechanical agitation inherent in the ‘standard’ washing procedures of suspension cells. Specifically, anti-gp120 antibodies that prevent interaction between gp120 and CD4 abrogate gp120-mediated reten-

tion of virus attached to suspension cells. Thus, under 'standard' washing procedures, the use of suspension cells as targets only permits the detection of viruses that are attached via the gp120–CD4 interaction, but does not permit the detection of viruses attached via CypA.

Here, we show that the use of adherent cells as targets for attachment assays is necessary to demonstrate the crucial role of CypA in HIV-1 attachment under 'standard' washing procedures. First, the high number of CypA-binding sites (heparans) on adherent cells results in an 8-fold increase of HIV-1 attachment compared with suspension cells for both gp120-deficient and wild-type viruses. Secondly, this 8-fold increase of attachment is observed using both CD4-positive and CD4-negative adherent HeLa cells (data not shown). Thirdly, neutralizing anti-gp120 antibodies that prevent the gp120–CD4 interaction do not affect viral attachment to CD4+HeLa adherent cells using 'standard' washing procedures. Fourthly, enzymatic removal of heparans completely abolishes HIV-1 attachment to adherent cells. Finally, anti-CypA IgG or an excess of exogenous CypA inhibits attachment of both gp120-deficient or wild-type viruses to adherent cells. Altogether, these results further demonstrate that the CypA–heparan interaction, but not the gp120–CD4 interaction, is necessary for the initial HIV-1 attachment to target cells.

Our work indicates that the choice of target cells for attachment assays is a critical parameter to define the respective roles of CypA and gp120 in HIV-1 adsorption. It is important to emphasize that the majority of studies examining HIV-1 entry utilized suspension cells as targets. Because suspension cells express low levels of heparans, their use as target cells in attachment assays does not permit the detection of viruses attached only via the CypA–heparan interaction using 'standard' washing procedures, but permits the detection of those subsequently attached via the gp120–CD4 interaction. In contrast, adherent cells that express high levels of heparans maintain viruses attached via CypA under 'gentle' or 'standard' washing procedures. It is important to note that viruses which lack CypA do not replicate in either suspension (peripheral blood lymphocytes or leukemic T cells) or adherent CD4+cells (CD4+HeLa cells), suggesting that our findings are applicable to a variety of cell systems.

#### **CypA mediates HIV-1 attachment to target cells via heparans**

In the search for CypA-binding sites on target cells, we found that CypA binds directly to heparans. First, we show that CypA binds to heparin *in vitro*. Furthermore, heparan derivatives such as heparin, heparan sulfate or dextran sulfate prevent this interaction. This is in accordance with a previous study which shows that another member of the cyclophilin family, CypB, binds directly to heparin (Denys *et al.*, 1998). Secondly, we demonstrate that CypA contains a heparin-binding domain located within its C-terminus. Introduction of mutations in this basic domain prevents CypA binding to heparans. Secondly, we show that CypA binds to target cells exclusively via heparans. Specifically, CypA does not bind to target cells upon enzymatic removal of cell surface heparans. Furthermore, a mutated form of CypA, which does not bind to heparan *in vitro*, fails to bind to target cells.

Finally, saturation of cell surface heparans with an excess of exogenous CypA prevents CypA binding to target cells. Altogether, these results suggest that CypA binds directly to cell surface heparans via its basic C-terminus.

Using several approaches, we demonstrate that HIV-1 attaches to target cells via the CypA–heparan interaction. First, we show that HIV-1 binds to heparin *in vitro* in a CypA-dependent manner. Specifically, wild-type or gp120-deficient viruses bind to heparin beads, whereas CypA-deficient viruses do not. This result suggests that CypA, but not gp120, is necessary for HIV-1 binding to heparin. Furthermore, exogenous recombinant wild-type CypA prevents HIV-1 binding to heparin beads, demonstrating that CypA directly mediates HIV-1 interaction with heparin. Secondly, we show that HIV-1 requires cell surface heparans to attach to target cells. Specifically, wild-type or gp120-deficient viruses fail to attach to target cells upon enzymatic removal of cell surface heparans. In addition, we observed a direct correlation between the surface levels of heparans on target cells and their capacity to attach HIV-1. Specifically, suspension cells that express low levels of heparans display a moderate capacity to attach HIV-1, whereas adherent cells that express high levels of heparans avidly attach HIV-1. Thirdly, addition of exogenous wild-type CypA that saturates the CypA-binding sites—the heparans—prevents both wild-type and gp120-deficient viruses from attaching to target cells. Importantly, these reduced levels of attachment correspond to those of CypA-deficient viruses. In contrast, a mutated form of CypA, which is unable to bind to heparin, does not affect HIV-1 attachment, demonstrating the specificity of the inhibition. Altogether, these results suggest that incorporated host CypA mediates HIV-1 attachment to target cells by interacting directly with cell surface heparans.

In the present study, we show that wild-type or gp120-deficient viruses are captured by anti-CypA IgG, whereas CypA-deficient viruses are not captured. Importantly, this capture is inhibited by an excess of recombinant human CypA, demonstrating the specificity of the immune interaction. Interestingly, we found that only a small portion of CypA is exposed to the extracellular medium. Furthermore, anti-CypA antibodies block HIV-1 infectivity by preventing attachment to target cells. Altogether, these results suggest that incorporated CypA is exposed at the surface of the virus, and thus may interact directly with cell surface heparans to permit HIV-1 attachment to target cells. Importantly, previous studies have demonstrated that cyclophilins are associated with or cross the plasma membrane. For example, CypA is secreted at very high levels ( $\mu\text{g/ml}$ ) in human blood (Allain *et al.*, 1995), and may be released into the extracellular medium upon cellular activation (Sherry *et al.*, 1992). Furthermore, a recent study using immunogold labeling demonstrated that CypA occurs at the plasma membrane (Uittenbogaard *et al.*, 1998). Similarly, another study showed that Myc-tagged CypB as well as endogenous, non-tagged CypB localizes to the extracellular surface of the plasma membrane of HeLa, COS and BHK cells (Price *et al.*, 1994). Finally, ninaA, the *Drosophila* homolog of human cyclophilins, is a membrane-bound protein (Stammes *et al.*, 1991). Although this particular cyclophilin homolog normally has a transmembrane domain, a truncated version of

this protein lacking this domain also localizes to the membrane (Stammes *et al.*, 1991). The fact that CypA does not contain a classical transmembrane domain suggests that other proteins may facilitate its translocation. A candidate class of proteins that may participate in this process are the heat shock proteins (hsps), which have been shown to be necessary for the cell surface exposure of other proteins (Vanbuskirk *et al.*, 1989; Morimoto *et al.*, 1990). Importantly, members of the hsp family have been shown to bind to CypA directly in an ATP-dependent manner, but independently of its peptidyl-prolyl *cis-trans* isomerase activity (Nadeau *et al.*, 1993). Interestingly, cell surface levels of hsps are significantly elevated upon HIV-1 infection (Di Cesare *et al.*, 1992). However, the mechanisms that control the release of CypA from Gag and its relocation to the viral surface remain to be investigated.

### Role of CypA in the HIV-1 life cycle

Previous studies suggest that CypA may act as an uncoating factor via its peptidyl-prolyl *cis-trans* isomerase activity (Braaten *et al.*, 1996; Luban, 1996). In this model, CypA, by interfering with CA-CA interactions, promotes the disassembly of the viral core within target cells. Although we cannot rule out this hypothesis, several lines of evidence argue against this model. First, the abundant cytosolic CypA within target cells does not rescue the infectivity of CypA-deficient virus derived from CsA-treated producer cells (Braaten *et al.*, 1996). Secondly, CypA does not influence assembly or destabilize the core within mature viral particles (Grättinger *et al.*, 1999; Wiegers *et al.*, 1999). Thirdly, recent data show that CypA is not associated with the core in mature virions (C.Aiken, unpublished results). Fourthly, a mutant virus (R9 G89V) that cannot bind CypA is nevertheless fully infectious during HIV-1 cell-to-cell transmission.

We propose an alternative model for the role of CypA in HIV-1 infectivity. In this model, CypA is a key mediator in the initial attachment of HIV-1 to the plasma membrane of target cells. Our present work demonstrates that CypA is essential for the initial attachment of the virus to target cells, whereas gp120 is responsible for subsequent steps in HIV-1 entry. Thus, we envision HIV-1 attachment as a multi-step process that requires the action of several virus-associated proteins. In the first step, viruses attach to target cells via an interaction between exposed CypA and heparans expressed at the surface of target cells. In a second step, once gp120 is in close proximity to the plasma membrane, it interacts with CD4 triggering the fusion process. Interestingly, a growing body of evidence points to the participation of cell surface heparans in viral attachment. Specifically, other viruses such as herpes simplex virus (HSV) (WuDunn and Spear, 1989), dengue virus (Chen *et al.*, 1997), respiratory syncytial virus (Krusat and Streckert, 1997), cytomegalovirus (Compton *et al.*, 1993), adeno-associated virus (Summerford and Samulski, 1998) and foot-and-mouth disease virus (Jackson *et al.*, 1996) all employ heparans for their initial step of infection. For example, the mode of entry of HSV is reminiscent of HIV-1. First, HSV binds to target cells via heparans (WuDunn and Spear, 1989), and is subsequently internalized via an interaction with a novel member of the tumor necrosis factor/nerve growth factor receptor (TNF/NGF) family (Montgomery *et al.*, 1996).

A fundamental question arises from our study: why does HIV-1 utilize CypA for its initial attachment to target cells? One possibility is that HIV-1 must enter cells rapidly to escape immune clearance. Since HIV-1 abundantly incorporates CypA (200 molecules per virus; Thali *et al.*, 1994), its attachment can occur via a large number of avidity interactions with heparans. Furthermore, this high avidity interaction is augmented by the subsequent gp120-CD4 interaction. Given that many different viruses utilize heparans for attachment, this common strategy may compensate for the limited endurance of some viruses within the host. Another advantage of using a host protein such as CypA is that the exposed site for viral attachment remains immunologically invisible. In this way, HIV-1 is a wolf in sheep's clothing, using host CypA to escape the host immune response. In conclusion, the present study demonstrates for the first time that a host protein, CypA, is a key mediator for the initial step of infection of a retrovirus—viral attachment.

## Materials and methods

### DNA constructs

The proviral clones R9 G89V and R9 G89A were a gift from D.Trono, whereas the R9  $\Delta$ gp160 plasmid clone was kindly provided by C.Aiken. By site-directed mutagenesis, the glycine in position 89 of CA within the CA-encoding region of plasmid R9 (NL4.3) (Gallay *et al.*, 1997) was changed to valine in the context of the proviral clone R9  $\Delta$ gp160, creating the double mutant R9 G89V  $\Delta$ gp160. Pseudotyping experiments were performed using DNA constructs encoding the amphotropic MLV envelope (MLV Env) and the VSV envelope (VSV Env) generously provided by D.Trono (Naldini *et al.*, 1996). Glutathione *S*-transferase-human CypA (GST-CypA) DNA construct (Luban *et al.*, 1993) was a generous gift from J.Luban. By site-directed mutagenesis, arginine148, lysine151, lysine154 and lysine155 located at the C-terminus of CypA were replaced by alanine in the context of the GST-CypA DNA construct.

### Transfections and infections

All viruses used in this study were transiently expressed by calcium phosphate transfection of 40  $\mu$ g of each proviral DNA construct as described previously (von Schwedler *et al.*, 1993). It is important to emphasize that wild-type and mutant viruses used in the present study were all produced from 293T cells, harvested at the same time and purified under the same conditions. Furthermore, only viruses derived from the same transfection were compared for attachment and infectivity assays. Viral supernatants, harvested 72 h post-transfection, were filtered through a 0.2  $\mu$ m-pore-size filter to remove cellular debris. The filtrate was concentrated with a 100-kDa-cutoff Centricon concentrator (Amicon) to eliminate free viral proteins such as CA, as well as free cellular proteins such as soluble proteoglycans, which would interfere with our assays. The virus was further purified on a 20–70% sucrose gradient. Cell-free infections were performed as previously described (Aiken and Trono, 1995). Briefly, virus (1 ng of p24) was added to adherent CD4+HeLa cells (40 000 cells). Forty-eight hours post-infection, infected cells were detected by X-Gal staining. Viral load was standardized by p24 antigen by ELISA (NEN-Dupont) or by RT using an exoRT assay (Aiken and Trono, 1995). CsA (Sigma) was used at a concentration of 10  $\mu$ M on virus producer cells. Cell-free infection was performed as described previously (von Schwedler *et al.*, 1993). For cell-to-cell infection, 293T cells transfected with proviral DNA constructs (48 h post-transfection) were washed extensively to remove free viruses and used as inoculum to infect adherent CD4+HeLa cells (40 000 cells). Cell-to-cell infection was subsequently processed as cell-free infection (see above). Cellular inoculum was standardized by p24 antigen in cell lysate of 293T transfected cells.

### Protein analyses and antibodies

Purification and immunoblot analysis of viruses produced from 293T transfected cells were conducted as previously described (von Schwedler *et al.*, 1993). Anti-MA and anti-gp41 IgG were purchased from Advanced Biotechnologies International. Anti-CA IgG, 2F5 anti-gp41 IgG and

F105 anti-gp120 IgG were obtained through the AIDS Research and Reference Program. Anti-gp120 IgG directed against the CD4-binding site of gp120; b12 (Burton *et al.*, 1991) and 15e (Robinson *et al.*, 1990) were a generous gift of P.Poignard, P.Parren and D.Burton. Rabbit anti-CypA serum was obtained by immunization with recombinant human CypA protein generated using the previously described GST-CypA plasmid (Luban *et al.*, 1993). Anti-CypA IgG was sequentially purified on CypA affinity and protein A columns.

#### Virus-capture assay

Ninety-six-well plates were coated for 16 h at 4°C with 5 µg/ml purified IgGs. Anti-gp120 IgG (b12 IgG1) (Burton *et al.*, 1991) was a generous gift of P.Parren and D.Burton. Plates were washed and blocked for 16 h with phosphate-buffered saline (PBS) containing 1% BSA. Purified viruses (10 ng of p24) from 293T transfected cells were incubated for 2 h at 37°C with the adsorbed IgG. Plates were washed, and bound viruses were lysed in PBS containing 0.5% NP-40. Amounts of p24 antigen in viral lysates were measured by p24 ELISA.

#### Subtilisin proteolytic analysis of viruses

Sucrose-gradient-purified viruses (R9) (100 µg of p24) from 293T cells were incubated for 2 h at 37°C with 3 mg/ml subtilisin (Sigma). Proteolysis reaction was stopped by the addition of 50 µM phenylmethylsulfonyl fluoride (PMSF) (Sigma). Protease-treated viruses were immediately pelleted through a 30% sucrose cushion. Viruses were resolved by SDS-PAGE (20% acrylamide) and the protein composition was determined by immunoblot analysis.

#### Attachment and internalization assays

The attachment assay was performed as follows: adherent CD4+HeLa cells ( $3 \times 10^6$  cells in a six-well plate) were incubated for 1 h at 4°C before viral exposure. Purified viruses (10 ng of p24) were added to target cells for 30 min at 4°C in a final volume of 2 ml of cold complete Dulbecco's modified Eagle's medium (DMEM). Adherent cells were then washed five times with 5 ml of cold PBS to remove unbound material, and lysed in PBS containing 0.5% NP-40. Under these conditions no internalization occurs, since no cytosolic p24 can be detected upon protease treatment of the target cells. As a viral internalization assay, the same conditions were used except that upon removal of the unattached virus, cells were transferred to 37°C for 2 h to allow viral internalization. Target cells were washed twice with 5 ml of PBS and trypsinized for 15 min at 37°C. Complete DMEM (10 ml) was used to neutralize the protease activity and the cells were further washed twice with 10 ml of PBS. Cells were lysed in PBS containing 0.5% NP-40. Attachment and internalization assays were monitored by measuring the amount of p24 and RT in the supernatant of cell lysates by ELISA and by an exoRT assay (Aiken and Trono, 1995), respectively.

#### Comparison between HIV-1 attachment to adherent and suspension cells

In order to compare the capacity of adherent and suspension cells to attach HIV-1, adherent CD4+HeLa cells were first detached using 10 mM EDTA for 30 min at 4°C. Cells were washed twice with PBS to remove EDTA and resuspended at  $3 \times 10^6$  cells in 2 ml of complete medium. Suspension CD4+H9 cells were washed twice with PBS and resuspended at  $3 \times 10^6$  cells in 2 ml of complete medium. Purified viruses (10 ng of p24) were added to target cells for 30 min at 4°C in a final volume of 2 ml of cold complete medium. For the 'standard' washing procedure, target cells were resuspended with a stream of 1 ml of cold PBS directly onto the pelleted cells using the fast setting of a pipet-aid. In contrast, for 'gentle' washing procedures, target cells were resuspended with the stream of buffer directed against the side of the tube using the slow setting of a pipet-aid. It is important to emphasize that the latter washing method resuspends the target cell pellet progressively, whereas the 'standard' method resuspends the pellet immediately. Resuspended cells were subsequently diluted to a final volume of 10 ml of cold PBS. These washing conditions were repeated five times. Finally, cells were lysed for 15 min in PBS containing 0.5% NP-40. Viral attachment was monitored by measuring the amount of p24 and RT in the supernatant of cell lysates by ELISA and by an exoRT assay (Aiken and Trono, 1995), respectively. Removal of cell surface heparans was performed by incubating target cells with heparinase (20 U/ml) (Sigma) for 2 h at 37°C. Cells were washed twice with PBS to remove heparinase.

#### CypA-binding to adsorbed heparin

Carbohydrate binding surface 96-well plates (Costar) were coated with 50 µg/ml heparin (Sigma) or BSA (Sigma) using the manufacturer's

recommended procedures. Recombinant human CypA (10 µg/ml) was incubated with adsorbed heparin for 1 h at room temperature in PBS containing 0.25% BSA and 0.05% Tween. Wells were washed 10 times with PBS to remove unbound CypA. Bound CypA was detected by ELISA using affinity-purified rabbit anti-CypA IgG (1 µg/ml). Competition experiments were performed by simultaneously adding CypA and an excess of heparin, heparan sulfate, dextran sulfate, DEAE-dextran and BSA (Sigma) (40 µg/ml).

#### CypA-binding to target cells

Adherent CD4+HeLa cells were detached using 10 mM EDTA for 30 min at 4°C. Cells were washed twice with PBS to remove EDTA and resuspended at  $3 \times 10^6$  cells in 2 ml of complete medium. Suspension CD4+H9 cells were washed twice with PBS and resuspended at  $3 \times 10^6$  cells in 2 ml of complete medium. Target cells were incubated with recombinant human CypA (50 µg) for 2 h at 4°C in PBS containing 0.5% BSA. Cells were washed twice with 3 ml of cold PBS to remove unbound CypA. Bound CypA was detected using affinity-purified anti-CypA IgG (10 µg/ml) in PBS containing 0.5% BSA for 1 h at 4°C and quantified by FACS. Removal of cell surface heparans was performed by incubating target cells with heparinase (20 U/ml) (Sigma) for 2 h at 37°C. Cells were subsequently washed twice with PBS to remove heparinase. Cell surface heparan levels were quantified by FACS analysis using anti-heparan sulfate IgM (10E4) (Seikagaku).

#### HIV-1-binding to heparin beads

Purified viruses (350 ng of p24) from 293T transfected cells were incubated for 2 h at 4°C with 50 µl of packed BSA- or heparin-Sepharose beads (Sigma). Beads were settled by gravity and washed twice with 1 ml of cold PBS. This settling by gravity avoids non-specific pelleting of viruses with the beads. Beads were subsequently washed eight times with 1 ml of cold PBS by low-speed centrifugation. Bound viruses were eluted by SDS gel loading buffer. Captured virus was analyzed by immunoblotting using anti-CA IgG (obtained through the AIDS Research and Reference Program). A competition experiment was performed by pre-incubation of beads with an excess of recombinant CypA for 2 h at 4°C. Unbound CypA was removed by washings prior to addition of viruses.

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